

Epigenetic Modifications: Are we Closer to Clinical Applicability?

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Abstract

Epigenetics encompass all inheritable, although potentially reversible changes in the genome, that do not alter the DNA code, but result from both developmentally (ensuring tissue specificity) and environmentally (resulting for exposure to many factors) driven modification of the spatial conformation of the DNA through chemical modification of the nucleotide chain itself or of the chromatin associated proteins. Epigenetic controls gene expression at a higher level than transcription, imprinting on the genome an environmental signature that is heritable through cell division and reflects a life-long experience. These types of modification (particularly DNA methylation patterns) explain phenotypic differences between identical twins. As opposed to genetic mutation, these modifications are reversible and are controlled by groups of enzymes (the epigenetic machinery, DNA methyltransferases (DNMT), histone deacetylases (HDACs) and histone acetyl transferases (HATs) and many more), hence, epigenetic marks include DNA methylation, histone modification and nucleosome positioning resulting in higher structural organisation of the chromatin regulating gene expression at the level of DNA accessibility for the transcriptional machinery to bind and initiate transcription.

Introduction:

Over the past few years, epigenetics has become an important field of research. Although epigenetic modifications to the genome do not involve a change in the nucleotide sequence, such alterations are important in all the processes that differentiate cells into several types contributing towards developing different tissues and patterns of epigenetic modification are transmitted to daughter cells with high fidelity and even across generations [1-3].

Several diseases have been related to alteration in such patterns and much research is now investigating how a variety of epigenetic mechanisms can be perturbed. The field has advanced predominantly in cancer, where perturbations include silencing of tumor suppressor genes and activation of oncogenes through change of CpG island methylation patterns, histone modifications, and dysregulation of DNA binding proteins. Such alteration in the epigenetic patterns are now considered as epigenetic mutations (epimutation) and are as important in tumorigenesis as genetic mutations, to the point that today cancer is thought to be the results of both genetic and epigenetic modifications [4-6]. Autoimmune diseases share a common origin in immunogenetic mechanisms. Epigenetic mechanisms are therefore also thought to be of importance as well in the pathogenesis of such complex diseases. DNA methylation has recently become the most widely studied mechanism in autoimmune diseases, with findings similar to those observed in cancer, notably in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [7].

DNA methylation

DNA methylations regulate an essential status of the chromatin (euchromatin or heterochromatin) by modulating nucleosomes spatial distribution and controlling DNA packaging through the binding of protein specifically recognizing methylated DNA and leading to chromatin compaction [8]. In humans, transcriptionally active genes

globally present non-methylated CpG island (s) preceding their promoter whereas the rest of CpG dinucleotides (50–70%) throughout the genome primarily in the heterochromatin regions are methylated towards silencing this part of the genome and contributing to chromosomal stability [9,10]. In contrast, in cancer or other diseases, the "healthy" CpG methylation profile of a cell is often inverted, almost mirrored [11] and tumor cells possess a globally hypomethylated genome, while at the same time focal hypermethylation is increased in specific places of the genome [4,12]. Islands preceding "anti-cancer" genes promoters are "closed" through hypermethylation, while oncogene promoters are "opened" by de-methylation. A number of genes have notably been implicated in cancer both genetically and epigenetically (the cyclin-dependent kinase inhibitor p16, the DNA repair genes MutL-homolog-1, O-6-methylguanine-DNA methyltransferase (MGMT) or breast cancer type 1 susceptibility protein, the cell cycle regulator Adenomatous polyposis coli [11,13]). On one hand, hypomethylation (which is estimated to be 20-50% less than normal in cancer) by disrupting the normal state of the chromatin, was shown to lead to chromosome instability by rendering accessible regions often containing retro-transposons (such as LINE-1 repeats), satellite DNA, and moderately repeated DNA sequences, whereas genes containing CpG clusters become hypermethylated, rendering them transcriptionally silent [4,12,14,15] This often includes promoter regions for genes themselves implicated in methylation (such as DNA methyltransferases) as is also observed in an inflammatory disease RA [16]. On the other hand, hypermethylation resulting in gene silencing can become so extensive that it has been tagged as addictive [17].

In SLE there is hypomethylation of promoter for genes overexpressed in the disease (Integrin, alpha L (ITGAL), CD40-Ligand, Perforin-1, CD70, Interferon gamma receptor 2, Matrix metalloproteinase-14, Lipocalin-2 [18-22] causing T-cell hyperactivity to perpetuate inflammatory responses [23-25], B cells to overexpress CD5 promoting autoimmunity [26]. In RA, synovial fibroblasts are

though to play a role in the initiation and perpetuation of the disease [27]. These cells were found to be globally hypomethylated resulting in the overexpression of inflammatory cytokines [28-30], similarly to LINE-1 elements [31,32]. On the other hand, hypomethylation of CpG islands in the IL-6 promoter gene resulting in overexpression contributes to B-cell responses [33,34]. Synovial cells resistance to apoptosis was also associated with hypermethylation of death receptor 3 (DR-3) gene [32,35-37]. In type 1 diabetes mellitus (T1DM) there is global hypermethylation related to altered metabolism of homocysteine in T-cells modulating their maturation and cytokine gene expression [38,39]. Multiple sclerosis (MS) is a unique case as the autoantigen (myelin basic protein) is known. The promoter of the peptidyl arginine deiminase type II (PAD2), the enzyme modifying myelin, is hypomethylated [40,41]. Systemic Sclerosis (SSc) is characterized by excessive collagen deposits in skin and other tissues. Hypermethylation of the Flil promoter, the transcription factor inhibiting collagen production is associated with this pathology [42,43].

Histone modification

DNA packaging includes wrapping around nucleosomes which can be more or less spatially tightened/loosen by post translational modification of the chromatin (DNA and histones proteins), allowing transcription to take place with more or less efficacy. Histones notably serve as building blocks to package DNA into higher or lower order of chromatin fibers and as such coordinate the changes between heterochromatin (tightly packed DNA "closed" to transcription) and euchromatin (exposed DNA, "open" for the binding of transcription factors [44]. Again, histone modifications (methylation, acetylation, phosphorylation, ubiquitination, sumoylation or biotinylation) follow a very specific pattern in healthy situation which can be altered by diseases. The loss of a particular modification (histone H4 lysine 16 acetylation (H4K16ac)) affects telomeres length during normal ageing and is targeted in cancer towards immortalising cells [11,45]. Both qualitative and quantitative changes in histone modifications are observed in cancer (much literature available), including many position and type of modification: H3K4me3, H3K4me2, H3ac are heavily enriched genome wide while H3K4me1 and H4ac shown reduced enrichment, H3K4me1 and H3K36me3 display aberrant distributions whereas H3K9me1, H3K20me1 and H3K27me1 show elevated levels of modification at specific gene loci and high levels of H3K27me2, 3, H3K79me3 H3K9me2, 3 were linked to gene repression or silencing). The expression of specific genes is also modulated at individual levels through such mechanisms in advanced cancers and metastasis either activating oncogenes (Myc) or silencing tumor suppressors (p53) [46-49]. Again, the machinery involved in making/erasing such modifications is often itself the target of dysregulation by these mechanisms (histone acetyltransferases, histone deacetylase) [11,50,51].

Nucleosomes are the primary inciting antigen in SLE. Histones are rendered immunogenic by the introduction of modifications (H3K4me3, H4K8, H3K27me3, H2BK12ac) during apoptosis, leading to the development of auto-antibodies [38,50-55]. In RA, the activity of HAT and HDAC is altered through histones acetylation resulting in matrix metalloproteinases and their regulators mediating cartilage destruction [56-58]. In mice models, histones hyperacetylation was associated with the induction of cell cycle arrest via p16 and p21 cyclin-dependent kinase inhibitors and a decrease in tumour necrosis factor- α synthesis the downregulation of hypoxia inducible factor and vascular endothelial growth factor ameliorating arthritis [58-60].

In T1DM, a few genes associated with autoimmunity and inflammation (cytotoxic T-lymphocyte-associated protein 4 (CTLA4), transforming growth factor-beta, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), mitogen-activated protein kinase p38, toll-like receptors, and Interleukin-6) were shown to exhibit dysregulated histone modification (H3K9me2) in lymphocytes [61-63]. Cardiovascular complications were also associated with Histone modifications (H3K4 and H3K9) [62-64]. In MS, increase in histone acetylation in the white matter was observed as well as hyperacetylation of histones in the promoter region of inhibitory genes involved in oligodendrocyte differentiation (Transcription factor 7-like 2, DNA-binding protein inhibitor ID-2, and sex determining region Y-box 2 (SOX2)) [65].

Nucleosome positioning

Nucleosome positioning patterns play an essential role in regulating gene expression. Nucleosomes too close to a transcription start sites (as small as 30 BP) may prevent transcription factors' to access the DNA hence, a nucleosome-free region need to allow the assembly of the transcription machinery [66]. Nucleosome positioning is itself regulated by as well as can influence DNA methylation [67]. This mechanism is important but has been studied in less detail so far. High-resolution genome-wide maps of nucleosome positions have so far provided much information about the organization of gene promoter and how this can facilitate or inhibit transcription [68]. Nucleosome position was notably shown to alter the pattern of expression of different splicing forms of genes responding to progesterone stimulation in breast cancer cells [69].

In RA, the binding of transcription factor NF- κ B to target gene appear altered by nucleosome positioning [70]. Difference in nucleosome distribution was also associated with genetic susceptibility to several autoimmune diseases (asthma, T1DM, Crohn's disease, cirrhosis) [70]. A polymorphism in region 17q12-q21 was associated with changes in expression of 2 genes (gasdermin B and ORM1-like 3 (ORMDL3)) resulting in allele-specific change in nucleosome distribution [70].

Technological advances

Analysing methylation at the genome level (epigenome-wide EWAS) is now relatively accessible. DNA methylation arrays technology is robust although it is dependent on the identification of CpG islands across the genome. The Human Methylation-450K CpG BeadChip kit has now been widely used, offering comprehensive coverage (96% of the known islands/promoters) and an affordable solution; however targeting only known promoters. The second generation EWAS kit (850K CpG) will offer coverage of promoters and enhancer, allowing more insight into to fine details of gene expression regulation. Microarrays may be replaced in the future by next generation sequencing technologies [71]; however, microarray are still very useful to pilot project or for large-scale experiments. The methylated DNA immunoprecipitation (MeDIP) technology [72] allows to immunoprecipitate single-stranded DNA fragments containing methylated DNA on a large scale for a chromosome or the whole genome. Combined with next-generation sequencing, MeDIP-Seq can stream down the analysis of the methylome by imputing methylation enriched DNA. Despite databases such as the GenomeStudio Browser displaying valuable information about chromosomal coordinates, island, percent GC, location in the CpG island allowing the analysis of the differential methylation between

samples of interest; it still remains quite difficult to analyse genome wide epigenetic data [73,74]. Tools are available; however they are much less advanced than in the genetics or gene expression field [75-77]. One of the main issues is that tissues are formed of different types of cells and each will have a different epigenetic signature, and unlike genetic information (static), epigenetics is changing with time (dynamic). New bioinformatics tools are also trying to combine GWAS data with EWAS data, to integrate genetic and environmental exposure information into disease context, however no publicly available databases for EWAS data have been set-up yet. The methylation profile of repeated element (ALU, LINE, SINE) mobile elements has not been fully completed and the impact of the repeat sequences has not been incorporated into the available tools to analyze EWAS.

Chromatin Immunoprecipitation (ChIP) represents the gold standard for analyzing protein based epigenetic marks [78]. It offers very specific information on proteins (histone or transcription factors (TF)) associated with a particular genomic region, such as a promoter. Due to the immunoprecipitation step, it looks at one particular modification or TF (targeted by the antibody) but can be coupled to PCR for one specific gene (as for biomarker development) or next generation sequencing to assess the overall role of that modification/TF. Histone profiling also requires high-quality monoclonal antibodies of which few are commercially available. A major effort by the NIH Epigenome Roadmap has launched a programme towards generating antibodies against all major histone modifications to be made readily available to investigators.

Epimutations: cause or consequences?

Distinct defects associated with epigenetic mechanisms are now widely reported in cancer (and start to be described in other diseases). Furthermore, many factors with long lasting association with diseases (mostly cancer); do not have mutagenic ability despite being classified as carcinogens (arsenite, chlorobenzene, nickel). These (as well as teratogens) were shown to exert their effect through epigenetic mechanisms [79, 80] providing further links between such mechanism and the occurrence of diseases.

These often target genes implicated in DNA repair as much as the epigenetic machinery itself. The question then arises whether they were an early event leading to carcinogenesis or a later consequence of the overall genetic instability. Point or larger size mutation are unlikely to be causing major cell function disruption if not associated with a mechanism allowing the development of the phenotype (cancer, other diseases) associated with the expression of these defective genes. DNA repair mechanisms are also challenged by the presence of methylated cytosine, creating a "lesion" that cannot easily be discriminated and resulting in an increase in G:T mismatch mutation. A conceptual argument was therefore made for an early involvement as a mean to lower the "proof reading" capability of cells, allowing DNA damage to persist unchecked. Accumulation of DNA damage thereafter can cause further increase in epimutation and vice versa [81]. Similar epimutations were also found in the area surrounding tumors suggesting a wider local perturbation possibly being the cause of an initial epigenetic event. If much descriptive work has demonstrated the presence and effect of such alterations, the precise nature of an original epigenetic switch is still elusive, in cancer as well as on other diseases although in the case of autoimmune disorders, inflammation appears a likely candidate. Nonetheless, the development of aberrant epigenomics was associated with all phases of cancer from initiation,

promotion, invasion, metastasis, and chemotherapy resistance [5,8,82-89] both at the genome wide and gene specific levels.

Epigenetic biomarkers

Since epigenetic marks are non-permanent, it was hypothesized that epigenomic profiles or specific epigenetic changes could be used to diagnose diseases, establish stages or predict response to treatment. Profiling methylation and acetylation with high accuracy is now a relatively "easy" approach thanks to recent advances in epigenomic analysis technologies. Epigenetic marks being defined as hallmarks of certain diseases, specific methylated CpG sites were investigated as biomarkers for diagnosis, staging, prognosis, and prediction of response to therapy [90]. For example hypermethylation silencing of certain loci (cadherin-13 (CDH13), myogenic regulator MYOD1, MGMT, cyclin-dependent kinase inhibitor p16-INK4b, glutathione S-transferase P, Ras association domain-containing protein 1, retinoic acid receptor RARB2, APC,) was specific to several cancer types [8,91,92] providing diagnostic value. Similarly, the hypermethylation of p16 and CDH13 has been associated with higher death risk and relapse [93]. Repetitive or mobile DNA elements (SINEs and LINEs) are hypomethylated in cancers [94], although to date the clinical utility of this remains unclear. Certain histone acetylation were also associated with poor prognosis [95,96], while other had diagnostic value [97]. Several chemotherapy drugs are attacking the genome although for cancer cell to die (by apoptosis), methylation and silencing of DNA repair mechanism must not have taken place yet hence, investigating these provides a means to predict response to treatment [98-101]. These observations still need to be reproduced and validated in large patient cohorts but implementation may not be far from clinical practice [96,102,103].

Epigenetic biomarker assays have therefore been developed by several companies. A test for the loci specific analysis of DNA-methylation levels was developed (Cygenia) for the complement component 1 subcomponent R (C1R) gene which is indicative of overall survival in acute myeloid leukemia (AML) [104]. Metastatic colorectal cancer patients with a lack of response to certain drug combination was associated with hypermethylation of the decoy receptor 1 (DCR1) gene [105]. Based on this a predictive biomarker test was introduced measuring levels of hypermethylation of the DCR1 gene. Further test will soon offer the possibility to measure the global methylation level at the DNA-methyltransferase 3A (DNMT3A) locus (epimutation) as a prognostic factor for AML [106] where both mutation and epimutations were associated with an elevated risk score and poor prognosis (patent pending). Another will quantify the abnormal shortening of telomeres epigenetically controlled at the PR domain containing-8 (PRDM8) gene in association with ageing and aplastic anemia or dyskeratosis congenital [105] Episona. The field is less advanced in other diseases although, the role of Th17 cells in autoimmune diseases has been established and quantifying these cells in the blood of patients with RA using a commercial assay detecting an epigenetic mark on the IL-17 gene was recently proposed as a diagnostic marker for RA [107].

Therapy targeting epigenetic mechanisms

Treatment (and prevention) strategies nowadays must take into consideration the role of epigenetic changes in the pathogenesis as well as progression of diseases. On the other hand, epigenetic changes are not permanently imprinted but result from enzymatic modifications of the DNA or histones. As such a therapeutic rationale was developed to

target such reversible changes. The epigenetic enzymes are the target of drug design for activity inhibitors mostly of the DNMT or HDAC. 5-azacytidine and 5-aza-2'-deoxycytidine are both nucleoside analogs and DNMT inhibitors. They showed much promises in in vitro model but due to the fact that these drugs affect the epigenome widely, that they cannot penetrate deeply inside solid cancer mass and have severe toxic side effects, they are now being replaced by other means of targeting DNMT, which are more specific and less toxic (anti-sense and small molecule) [108,109]. In clinical trials, HDAC inhibitors (butyrate, trichostatin A (TSA), depsipeptide, oxamflatin, MS-275) showed better tolerability and more activity with objective tumor regression [110,111]. They appear to induce the expression of regulators of the cell-cycle, causing cell-cycle arrest [110,112]. A combination of DNMT and HDAC inhibitors has been proposed as a novel approach for therapeutic intervention as it showed a synergistic reactivation of tumor suppressor genes and an enhanced antineoplastic effect against tumor cells [95]. Procainamide and hydralazine are two drugs used in SLE that ultimately result in the inhibition of DNA methylation through different pathways [113-119].

Epigenetic is often associated with the quote "We are what we eat and breath" as a recognition of the impact of our diet and environment on our health [120]. Relationships between these factors and cancer and other diseases have been clearly established [94,121,122]. Glucose and insulin levels are factors regulating methylation by modulating the activity of DNMT [123-125]. Several components of our diet were shown to have inhibitory activity on the epigenetic machinery such as dietary chemopreventive agents (butyrate, diallyl disulfide, sulforaphane) on HDAC [126], a plant molecule (resveratrol) on Sirtuin-1 [127] and green tea (polyphenols and phenethyl isothiocyanate) on both DNMT and HDAC [128,129]. On the other hand, transposons and other mobile genetic elements can be activated by different types of environmental stress including dietary stress [130-131].

Conclusion

The underlying goals for most epigenetic research is both to understand the role of epigenetic mechanisms in health and diseases and ultimately, to develop biomarkers or therapeutic interventions. Epigenetic has certainly delivered many new insights into the understanding of the initiation, progression and response to therapy for diseases such as cancer and autoimmune conditions. Recent advances in technologies are with no doubts an important factor in this and will certainly continue to contribute novel development in the biomarker field. The therapeutic potential of novel HDAC and DNMT inhibitors is promising and following from the genetic and then the transcriptomic and proteomic eras, epigenetic may now offer the missing link allowing the integration of our life experience into our health.

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